REMARKS

The only difference between the substitute specification submitted herewith and that which was required to be filed in the parent application Serial No. 09/660,522 and which was entered is that the word "myocardia" at page 10, line 7 of the original English application has not been changed. No new matter is added to the substitute specification.

New claims 7 and 8 are directed to a method for treating a cardiovascular disease via intracoronary injection of HGF vector, and find support in the specification at page 9, lines 27 to page 10, line 13, and in Example 8. Both claims were allowed in parent application Serial No. 09/660,522. Claim 9 recites the specific diseases that can be treated with the claimed method. Claim 12 finds support in the specification at page 10, line 7, and in the fact that the word "myocardia" at page 10, line 7, should be "cardiomyopathy."

A declaration by a translator explaining this mistranslation will be filed within the next few months.

Accordingly, no question of new matter arises and entry and consideration of this Amendment are requested, respectfully.

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Respectfully submitted,

Susan J. Mack

Susan J. Mack
Registration No. 30,951

SUGHRUE MION, PLLC
Telephone: (202) 293-7060
Facsimile: (202) 293-7860
WASHINGTON OFFICE
23373
PATENT TRADEMARK OFFICE

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DESCRIPTION

MEDICAMENT COMPRISING HGF GENE

Technical Field to which the Invention Pertains

[01] The present invention relates to a medicament for use in ~~the~~ gene therapy and the like. More particularly, the present invention relates to a medicament comprising a hepatocyte growth factor (HGE) gene as well as a liposome containing the HGF gene.

Prior Art~~s~~

[02] HGF is a physiologically active peptide that exhibits diverse pharmacological activities. The pharmacological activities of HGF are described in, e.g., JIKKEN-IGAKU (Experimental Medicine), Vol. 10, No. 3 (extra issue), 330-339 (1992). In view of its pharmacological activities, HGF is expected to be useful as: a drug for the treatment of ~~liver~~ cirrhosis of the liver or renal diseases; epithelial cell growth accelerators; anti-cancer agents; agents for the prevention of side effects in cancer therapy; agents for the treatment of lung disorders, gastrointestinal damages or cranial nerve disorders; agents for the prevention of side effects in immunosuppression; collagen degradation accelerators; agents for the treatment of cartilage disorders, arterial diseases, pulmonary fibrosis, hepatic diseases,

blood coagulopathy, plasma hypoproteinosis or wounds; agents for the improvement of nervous disorders; hematopoietic stem cell potentiators; and hair growth promoters (Japanese Patent KOKAI (Laid-Open) Nos. 4-18028 and 4-49246, EP 492614, Japanese Patent KOKAI (Laid-Open) No. 6-25010, WO 93/8821, Japanese Patent KOKAI (Laid-Open) Nos. 6-172207, 7-89869 and 6-40934, WO 94/2165, Japanese Patent KOKAI (Laid-Open) Nos. 6-40935, 6-56692 and 7-41429, WO 93/3061, Japanese Patent KOKAI (Laid-Open) No. 5-213721, etc.).

[03] As to gene therapy, extensive studies and investigations recently have been ~~currently~~ made all over the world for treatment of adenosine deaminase deficiency, AIDS, cancer, pustulous fibrosis or hemophilia, etc.

[04] However, gene therapy using ~~the~~ HGF genes is unknown yet. It is even unclear if such gene therapy ~~is effectively applicable~~ will be effective.

Problems to be solved by the Invention

[05] HGF is known to be one of the drugs that have a short half life in blood. As a result ~~natural consequence~~, persistent topical administration ~~has been desired~~ for HGF would be desirable.

[06] In view of the diverse pharmacological activities of HGF, HGF is expected to be developed as a drug having extensive ~~extended~~ applications to various diseases. On the other hand, when HGF is systemically administered, side effects might be caused due to the diverse

pharmacological activities of HGF. In addition, when HGF itself is intravenously administered, HGF ~~encounters such a~~ has the drawback that a considerable amount of HGF is retained in the liver, resulting in reduction of the amount of HGF ~~to~~ that reaches the target organ.

[07] <u>Means for solving the Problems</u>

The present invention has been made to solve the foregoing problems. In summary, the present invention relates to:

(1) a medicament comprising a HGF gene;

(2) a liposome containing the HGF gene;

(3) a liposome according to (2), which is a membrane fusion liposome fused to Sendai virus;

(4) a medicament comprising the liposome according to (2) or (3);

(5) a medicament according to (1) or (4), for use in the treatment ~~for~~ of arterial disorders; and,

(6) a medicament according to (1) or (4), for use in the treatment ~~for~~ of cartilage injuries.

<u>Brief Description of the Drawings</u>

[08] Fig. 1 shows ~~an~~ expression of HGF in rat coronary endothelial cells sensitized with the hemagglutinating virus of Japan (HVJ)-liposome-DNA in Test Example 1.

[09] In Fig. 2, the (line) graph shows ~~a~~ cell growth rate in the presence or absence of HGF from the

HVJ-liposome-cont-sensitized endothelial cells in Test Example 2, wherein "DSF" designates a group of ~~the~~ endothelial cells sensitized with HVJ-liposome-cont and "HGF" designates a group of endothelial cells incubated in the presence of recombinant human HGF ~~in~~ at a predetermined concentration. The bar in Fig. 2 shows ~~a~~ the cell growth rate of the HVJ-liposome-DNA-sensitized endothelial cells in Test Example 2, wherein "DSF" designates a group of ~~the~~ endothelial cells sensitized with HVJ-liposome-cont and "HGF vector" designates a group of ~~the~~ endothelial cells sensitized with HVJ-liposome-DNA.

[10] Fig. 3 shows ~~a~~ the cell growth rate of endothelial cells sensitized with HVJ-liposome-DNA in the presence or absence of anti-HGF antibody in Test Example 2, wherein "control" represents a group of ~~the~~ HVJ-liposome-cont-sensitized endothelial cells incubated in the presence of IgG control; "HGF" represents a group of ~~the~~ HVJ-liposome-DNA-sensitized endothelial cells incubated in the presence of IgG control; and "HGFab" represents a group of ~~the~~ HVJ-liposome-DNA-sensitized endothelial cells incubated in the presence of ~~a~~ rabbit anti-human HGF antibody. The cell growth rate (%) is expressed in terms of relative % when the growth rate in the control group is ~~made~~ set as 100.

[11] Fig. 4 is a graph showing the cell growth effect of ~~the~~ culture supernatant from ~~the~~ HVJ-liposome-DNA-sensitized rat vascular smooth muscle cells (hereinafter often abbreviated as VSMCs) on rat coronary

endothelial cells in Test Example 3, wherein "control" designates a group to which was added ~~with the~~ culture supernatant from the HVJ-liposome-cont-sensitized rat VSMCs, and "HGF" designates a group to which was added ~~with the~~ culture supernatant from the HVJ-liposome-DNA-sensitized rat VSMCs.

[12] Fig. 5 is a graph showing the results from ~~in~~ Test Example 3 in which the concentration of HGF in the supernatant from ~~the~~ incubated HVJ-liposome-DNA-sensitized rat VSMCs was determined using ~~the~~ anti-human HGF antibody. In the figure, "no-treatment" represents a group to which was added ~~of the~~ culture supernatant from ~~of~~ non-sensitized VSMCs; "control" represents a group to which was added ~~of the~~ supernatant from the incubated HVJ-liposome-cont-sensitized rat VSMCs; and "HGF" represents a group to which was added ~~of the~~ supernatant from the incubated HVJ-liposome-DNA-sensitized rat VSMCs.

[13] Fig. 6 is a graph showing the results from ~~in~~ Test Example 3 in which the concentration of HGF in the supernatant from the incubated HVJ-liposome-DNA-sensitized rat VSMCs was determined using ~~the~~ anti-rat HGF antibody. In the figure, "no-treatment" represents a group treated with ~~of~~ the culture supernatant of non-sensitized VSMCs; "control" designates a group treated with ~~of~~ the supernatant from the incubated HVJ-liposome-cont-sensitized rat VSMCs; and "HGF" designates a group treated with ~~of~~ the supernatant from the incubated HVJ-liposome-DNA-sensitized rat VSMCs.

[14] Fig. 7 is a graph showing the cell growth effect of the supernatant from ~~the~~ incubated HVJ-liposome-DNA-sensitized rat coronary endothelial cells on rat coronary endothelial cells in Test Example 4, wherein A, B and C designate, respectively, a group to which was added ~~with the~~ supernatant ~~of the~~ from incubated HVJ-liposome-DNA-sensitized rat coronary endothelial cells, a group to which was added ~~with the~~ supernatant ~~of the~~ from incubated HVJ-liposome-cont-sensitized rat coronary endothelial cells, and a group of no-treatment animals.

[15] Fig. 8 shows the cell growth effect of HVJ-liposome-DNA-sensitized rat coronary endothelial cells on rat coronary endothelial cells in the presence of an anti-HGF antibody in Test Example 4. In the figure, A represents a group to which was added ~~with the~~ supernatant from the incubated HVJ-liposome-DNA-sensitized rat coronary endothelial cells; B represents a group to which was added ~~with the~~ supernatant from the incubated HVJ-liposome-cont-sensitized rat coronary endothelial cells; C represents a group to which was added ~~with the~~ anti-HGF antibody ~~to the~~ supernatant from the incubated HVJ-liposome-DNA-sensitized rat coronary endothelial cells and wherein was added to the supernatant; and D represents a group to which was added ~~with the~~ control antibody ~~to the~~ supernatant ~~of~~ from the incubated HVJ-liposome-DNA-sensitized rat coronary endothelial cells, wherein was added to the supernatant.

[16] Fig. 9 is a drawing showing ~~the~~ cell growth of endothelial cells in Test Example 5 when HVJ-liposome-DNA-sensitized human VSMCs were co-incubated with

non-sensitized human endothelial cells. In the figure, "control" represents a group of human endothelial cells co-incubated with HVJ-liposome-cont-sensitized VSMCs, and "HGF" represents a group of human endothelial cells co-incubated with the supernatant from the incubated HVJ-liposome-DNA-sensitized VSMCs.

[17] Fig. 10 indicates the cell growth of endothelial cells in Test Example 6 when HVJ-liposome-DNA-sensitized rat VSMCs were co-incubated with non-sensitized rat coronary endothelial cells. In the figure, "control" represents a group of human endothelial cells co-incubated with the HVJ-liposome-cont-sensitized VSMCs, and "HGF" represents a group of human endothelial cells co-incubated with the culture supernatant ~~of~~ from the HVJ-liposome-DNA-sensitized VSMCs.

[18] Fig. 11 shows an increase in the number of minute blood vessels in rat heart muscle directly injected with HVJ-liposome-DNA in Test Example 8, wherein "HGF" denotes the number of minute blood vessels in rat heart muscle directly injected with HVJ-liposome-DNA, and "control" denotes the number of minute blood vessels in rat heart muscle directly injected with HVJ-liposome-cont.

[19] Fig. 12 is a drawing showing that 3 weeks after administration of HVJ-liposome-DNA into the joint, development of cartilage-like cells was noted in Test Example 9, ~~in which the~~ as evidenced by synthesis of Toluidine Blue-stained proteoglycan ~~was observed~~.

[20] Fig. 13 is a drawing showing that 4 weeks after administration of HVJ-liposome-DNA into the joint,

development of cartilage-like cells was noted in Test Example 9, ~~in which the~~ as evidenced by synthesis of Toluidine Blue-stained proteoglycan ~~was observed~~.

[21] Fig. 14 is a drawing showing that even 4 weeks after administration of HVJ-liposome-DNA (TGF-β) prepared in Comparative Example 2 into the joint, ~~such~~ development of cartilage-like cells as evidenced by the fact that synthesis of ~~observing~~ Toluidine Blue-stained proteoglycan ~~synthesis~~ was not observed ~~noted~~ in Test Example 9.

[22] Fig. 15 is a drawing showing that even 4 weeks after administration of HVJ-liposome-cont prepared in Comparative Example 1 into the joint, no such development of cartilage-like cells as evidenced by synthesis of ~~observing~~ Toluidine Blue-stained proteoglycan ~~synthesis~~ was observed ~~noted~~ in Test Example 9.

Best Mode for carrying out the Invention

[23] The "HGF gene" employed in the present invention means ~~indicates~~ a gene capable of expressing HGF. Thus, so long as the ~~a~~ polypeptide expressed has substantially the same effect as that of HGF, the HGF gene may have a partial deletion, substitution or insertion of the nucleotide sequence, or may have other nucleotide sequences ligated therewith at the 5'-terminus and/or 3'-terminus thereof. Typical examples of such HGF genes include HGF genes as described in Nature, 342, 440 (1989), Japanese Patent KOKAI (Laid-Open) No. 5-111383, Biohem. Biophys. Res. Commun., 163, 967 (1989), etc.

These genes may be used in the present invention.

[24] The HGF gene is incorporated into an appropriate vector and the HGF gene-bearing vector is provided for use. For example, the HGF gene may be used in the form of a viral vector having the HGF gene as described hereinafter, or in the form of an appropriate expression vector having the HGF gene.

[25] The "pharmaceutical composition" used in the present invention ~~indicates~~ means a medicament for the treatment or prevention of human diseases, which is attributed to the pharmacological activities of HGF. For example, exemplified are medicaments for the treatment or prevention of the diseases given hereinabove.

[26] According to the present invention, the HGF gene is introduced into cells wherein HGF is expressed in those cells to exhibit the pharmacological actions. Thus, the medicament of the present invention is effectively applicable to the diseases for which HGF itself is effective.

[27] Where the HGF gene is introduced into, e.g., cells, the growth of vascular endothelial cells is accelerated, while undesired growth of vascular smooth muscle cells is not accelerated, as demonstrated in the Examples hereinafter. Moreover, as demonstrated in the Examples hereinafter, where the HGF gene is introduced into the heart in in vivo animal tests using rats, angiogenesis is observed. Therefore, the HGF gene is effective for the treatment and prevention of arterial

disorders, in particular, various diseases caused by a disturbance which mainly involves abnormal proliferation of vascular smooth muscle cells (e.g., restenosis after percutaneous transluminal coronary angioplasty (PTCA), arteriosclerosis, insufficiency of peripheral circulation, etc.), and for the treatment and prevention of diseases such as myocardial infarction, myocardia, peripheral angiostenosis, cardiac insufficiency, etc. HGF itself is also useful for the treatment and prevention of the diseases as described above, since HGF promotes the proliferation of vascular endothelial cells but does not promote the growth of vascular smooth muscle cells. The pharmacological effects of the HGF gene are attributed to those of HGF itself.

[28] As demonstrated in the Examples hereinafter, introduction of the HGF gene into the joint results in promoting repair of articular cartilage cells thereby to promote the proliferation of proteoglycan-synthesizing cells. Therefore, the HGF gene is effective for the prevention and treatment of various cartilage injuries such as osteogenetic abnormality, arthritis deformans, discopathy deformans, fracture repair and restoration insufficiency, trauma caused by sport~~ing~~s, key puncher's disease, etc. HGF itself is useful for the treatment and prevention of the diseases described above, since HGF promotes repair and growth of cartilage cells. The effects of the HGF gene are based on those of HGF itself.

[29] A "liposome" is a closed vesicle of lipid bilayer encapsulating an aqueous compartment ~~therein~~. It is known that the lipid bilayer membrane structure is extremely similar to biological membranes. To prepare the liposomes of the present invention, phospholipids are employed. Typical examples of phospholipids are phosphatidylcholines such as lecithin, lysolecithin, etc.; acidic phospholipids such as phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, phosphatidylic acid, etc.; or phospholipids obtained by replacing an acyl group(s) of these acidic phospholipids with lauroyl, myristoyl, oleoyl, etc.; and sphingophospholipids such as phosphatidylethanolamine, sphingomyelin, etc. Neutral lipids such as cholesterol may also be added to these phospholipids. The liposomes may be prepared, in a conventional manner, from naturally occurring materials such as lipids in normal cell membranes. The liposomes containing the HGF gene of the present invention may be prepared, for example, by suspending a thin layer of purified phospholipids in a solution containing the HGF gene and then treating the suspension in a conventional manner, such as by ultrasonication.

[30] The liposomes containing the HGF gene of the present invention may be appropriately fused to viruses, etc. to form membrane fusion liposomes. In this case, it is preferred to inactivate viruses, e.g., through ultraviolet irradiation, etc. A particularly preferred

example of the membrane fusion liposome is a membrane fusion liposome fused with Sendai virus (hemagglutinating virus of Japan: HVJ). The membrane fusion liposome may be produced by the methods ~~as~~ described in NIKKEI Science, April, 1994, pages 32-38; J. Biol. Chem., 266 (6), 3361-3364 (1991), etc. In more detail, the HVJ-fused liposome (HVJ-liposome) may be prepared, e.g., by mixing purified HVJ inactivated by ultraviolet irradiation, etc. with a liposome suspension containing the HGF gene vector, gently agitating the mixture and then removing unbound HVJ by sucrose density gradient centrifugation. The liposomes may be bound to substances having an affinity to target cells, thereby ~~to~~ enhanc~~e~~ing ~~an~~ the efficiency of gene introduction into the target cells. Examples of ~~the~~ substances having an affinity ~~to~~ for ~~the~~ target cells include ligands such as an antibody, a receptor, etc.

[31] For introduction of the HGF gene into cells, conventional methods are employed, which are roughly classified into introduction via viral vectors and other strategies (NIKKEI Science, April, 1994, pages 20-45; GEKKAN YAKUJI, 36 (1), 23-48 (1994) and references cited therein). Both types of methods ~~are available~~ can be used for ~~the~~ preparation of the medicament of the present invention.

[32] The former type of method using viral vectors comprises the step of incorporating the HGF gene into, e.g., a retrovirus, an adenovirus, an adeno-related virus, a herpes virus, a vaccinia virus, a poliovirus, a

sindbis virus or other RNA virus~~es~~. Of these viruses, a retrovirus, an adenovirus and an adeno-related virus are particularly preferably employed for ~~the~~ introduction.

[33] Examples of the other methods include the liposome method, lipofectin method, microinjection method, calcium phosphate method, electroporation method. Of these methods, particularly preferred is the liposome method.

[34] For practical use of the HGF gene as a medicament, it is advantageous to introduce the HGF gene directly into the body (<u>in vivo</u> method). Alternatively, certain cells are collected from the human, the HGF gene is then introduced into the cells outside the body, and ~~the HGF gene-introduced~~ cells having the HGF gene introduced therein are returned to the body (<u>ex vivo</u> method). These methods are described in NIKKEI Science, April, 1994, pages 20-45; GEKKAN-YAKUJI, 36 (1), 23-48 (1994) and references cited therein. Any of these methods ~~are~~ is suitabl~~y~~e, ~~chosen~~ depending upon ~~a~~ the disease to be treated, target organs, etc. and may be applied to the medicament compositions of the present invention.

[35] The <u>in vivo</u> method is less costly, less laborious and therefore more convenient than the <u>ex vivo</u> method, but the latter method provides a higher efficiency of introduction of the HGF gene into cells.

[36] Where the medicament of the present invention is administered by the <u>in vivo</u> method, the medicament may be administered through any route appropriate for diseases to be treated, such as via target organs, etc. The

medicament may be administered intravenously, intra-arterially, subcutaneously, intramuscularly, etc., or directly to the objective organ of the disease~~s~~, e.g., kidney, liver, lung, brain, nerve, etc. Direct administration to the objective site can treat the target organ selectively. For example, in gene therapy using a gene for restenosis after PTCA, the composition may be administered intraarterially (JIKKEN-IGAKU, 12 (extra issue 15), 1298-1933 (1994). Preferably, the medicament of the present invention is applied at the tip of a balloon used for PTCA and ~~rubbed~~ the tip is rubbed against a blood vessel, whereby the medicament may be introduced directly into vascular endothelial cells and vascular smooth muscle cells.

[37] Where the *ex vivo* method as described above is used to introduce the HGF gene, human cells (e.g., lymphocytes or hematopoietic stem cells) are harvested in a conventional manner, and the harvested cells are sensitized with the medicament of the present invention for gene introduction. Thereafter the HGF-producing cells are inserted back ~~to~~ into the human.

[38] Where the medicament is administered by the *in vivo* method, the medicament may take various preparation forms, including the form of a liquid preparation. In general, the medicament may be preferably prepared as ~~into~~ an injection comprising the HGF gene as an active ingredient. If necessary and desired, conventional carriers may be added to the composition. The injection

may be prepared in a conventional manner, e.g., by dissolving the HGF gene in an appropriate solvent (e.g., sterilized water, a buffered solution, a physiological saline solution, etc.), filtering the solution through a filter, etc. for sterilization, filling up with the solution ~~in~~ a sterile container. The medicament may be prepared using the HGF gene-~~incorporated~~ containing viral vector, instead of the HGF gene itself. Where the liposomes containing the HGF gene embedded therein (or HVJ-liposomes) are employed, the medicament may be in the form of liposome preparations such as a suspension, a frozen preparation, a centrifugally concentrated frozen preparation, etc.

[39] The content of the HGF gene in the medicament may be appropriately varied depending upon diseases to be treated, target organs, patients' ages or body weights, etc. However, it is appropriate to administer in a dose of 0.0001 mg to 100 mg, preferably 0.001 mg to 10 mg when calculated as the HGF gene. The dose may be divided into several days or a few months.

Examples

[40] Hereinafter the present invention will be described in more detail with reference to the examples but is not deemed to be limited thereto. Materials and methods used in the following examples are outlined below.

Materials and methods

(1) HGF expression vector

[41] The HGF expression vector was prepared by inserting human HGF cDNA (2.2 kb, Biochem. Biophys. Res. Commun., 172, 321-327 (1990); Japanese Patent KOKAI (Laid-Open) No. 5-111383) between the EcoRI and NotI sites of a pUC-SRα expression vector (FEBS, 333, 61-66 (1993)). In this plasmid vector, transcription of HGF cDNA is regulated by the SRa promoter (Nature, 342, 440-443 (1989)).

(2) Cell culture

[42] Rat coronary endothelial cells were isolated from ~~the~~ enzymatically digested heart of 8-week~~s~~ old ~~aged~~ Sprague-Dawley (SD) rats by density gradient centrifugation (Transplantation, 57, 1653-1660 (1994)). Rat aortic vascular smooth muscle cells (VSMCs) were obtained from 12 week~~s~~ old ~~aged~~ SD rats by enzymatic treatment (J. Clin. Invest., 93, 355-360 (1994)). These cells were maintained in DMEM medium supplemented with 10% (vol/vol) calf fetal serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were incubated at 37°C in a humidified 95% air-5% CO_2 atmosphere. The culture medium was routinely changed at 2 day-intervals. Both immunopathological and morphological observation revealed that these cells were endothelial cells and smooth muscle cells, respectively.

[43] Human aortic endothelial cells (five passages)

and human VSMCs (five passages) were obtained from Kurabo Co. The endothelial cells were incubated in a manner similar to the above method in MCDB131 medium supplemented with 5% calf fetal serum, epidermal growth factor (10 ng/ml), basic fibroblast growth factor (2 ng/ml) and dexamethasone (1 μM).

[44] Endothelial cells in the stationary state were prepared according to the method described in J. Clin. Invest., 86, 1690-1697 (1990), ibid., 94, 824-829 (1994).

(3) Transfection of the HGF gene into HVJ-liposomes in vitro

[45] Endothelial cells or VSMCs were inoculated for sensitization on a 6-well plate in a cell count of 10^6 and proliferated to reach 80% confluence. The cells were washed 3 times with a balanced salt solution (137 mM NaCl, 5.4 mM KCl, 10 mM Tris-HCl, pH 7.6; hereinafter abbreviated as "BSS") supplemented with 2 mM calcium chloride. To the cells was added 1 ml of a solution of the HVJ-liposome-DNA (containing 2.5 mg of lipids and 10 mg of the embedded DNA) obtained in Example 1 hereinafter or 1 ml of a solution of the HVJ-liposome-cont obtained in Comparative Example 1 hereinafter. The resulting mixture was incubated at 4°C for 5 minutes and at 37°C for a further 30 minutes. The cells were washed and maintained in a fresh medium containing 10% bovine serum in a CO_2 incubator.

(4) Assay for the HGF concentrations in endothelial cells and VSMCs

[46] The concentration of HGF produced from the sensitized endothelial cells and VSMCs was assayed by ELISA. That is, rat or human endothelial cells or VSMCs were inoculated on a 6-well plate (made by Corning) in a cell density of 5×10^4 cells/cm^2, followed by incubation for 24 hours. The medium was replenished 24 hours after the sensitization, and incubation was continued for a further 48 hours. To investigate if HGF was released, the sensitized cells (48 hours after sensitization) were washed and added to 1 ml of a serum-free medium containing 5×10^{-7} M insulin, 5 μg/ml transferrin and 0.2 mM ascorbate. After 24 hours, the culture media was ~~were~~ collected, centrifuged at 600 g for 10 minutes and then stored at -20°C.

[47] The HGF concentration in the media was determined by an enzyme immunoassay using an anti-rat HGF antibody or an anti-human HGF antibody (Exp. Cell Res., 210, 326-335 (1994); Jpn. J. Cancer Res., 83, 1262-1266 (1992)). A rabbit anti-rat or an anti-human HGF IgG was coated onto a 96-well plate (made by Corning) at 4°C for 15 hours. After blocking with 3% bovine serum albumin-containing PBS (phosphate buffered saline), the culture medium was added to each well, and incubation was performed at 25°C for 2 hours. After washing each well 3 times with PBS containing 0.025% Tween (PBS-Tween), a biotinated rabbit anti-rat HGF IgG

or an anti-human HGF IgG was added to each well followed by incubation at 25°C for 2 hours. After washing with PBS-Tween, each well was incubated together with horse radish peroxidase-bound streptoavidin-biotin complex (PBS-Tween solution). The enzymatic reaction was initiated by adding thereto a substrate solution (containing 2.5 mM o-phenylenediamine, 100 mM sodium phosphate, 50 mM citrate and 0.015% hydrogen peroxide). The reaction was terminated by adding 1 M sulfuric acid to the system. Absorbance was measured at 490 nm. The anti-human HGF antibody is ~~cross-~~reactive only with human HGF, ~~but~~ and not with rat HGF. The anti-rat HGF antibody is ~~cross-~~reactive solely with rat HGF, ~~but~~ and not with human HGF.

<u>(5) HGF</u>

[48] The human and rat recombinant HGFs employed were purified from the culture solution of CHO cells or C-127 cells transfected with an expression plasmid bearing human or rat HGF cDNA (Cell, <u>77</u>, 261-271 (1994); J. Clin. Invest., <u>93</u>, 355-360 (1994)).

<u>(6) Statistical analysis</u>

[49] All runs were repeated at least 3 times. Data measured are shown by mean ± standard error. Statistical analysis of the measured data was made according to ~~the~~ Duncan's test.

(7) Hematoxylin-Eosin (HE) staining and Azan staining

[50] Ten days after the gene introduction, the rats having the HGF gene-introduced therein ~~rats~~ were sacrificed by perfusion with heparinized physiological saline. Fixation was then made overnight with a 4% paraformaldehyde PBS solution. After fixation, the tissue was embedded in paraffin. Slides were prepared and stained with HE and Azan in a conventional manner. The slides were examined on a microscope to count the number of microvessels.

Example 1

Preparation of HVJ-liposomes containing the HGF expression vector

[51] Phosphatidylserine, phosphatidylcholine and cholesterol were mixed with tetrahydrofuran in a weight ratio of 1 : 4.8 : 2. By distilling tetrahydrofuran off through a rotary evaporator, the lipid mixture (10 mg) was precipitated onto the container wall. After 96 µg of high mobility group (HMG) 1 nuclear protein purified from bovine thymus was mixed with a BBS (200 µl) solution of plasmid DNA (300 µg) at 20°C for an hour, the mixture was added to the lipid mixture obtained above. The resulting liposome-DNA-HMG 1 complex suspension was mixed with a vortex, ultrasonicated for 3 seconds and then agitated for 30 minutes.

[52] The purified HVJ (strain Z) was inactivated by UV irradiation (110 erg/mm^2 sec) for 3 minutes immediately before use. BSS was added to and mixed with the

liposome suspension (0.5 ml, containing 10 mg of the lipids) obtained above and HVJ (20,000 hemagglutinating units) to make the ~~whole~~ total volume 4 ml. The mixture was incubated at 4°C for 10 minutes and gently agitated at 37°C for a further 30 minutes. The unreacted HVJ was removed from the HVJ-liposomes by sucrose density gradient centrifugation. That is, the upper layers in the sucrose density gradient were collected to give the HVJ-liposomes containing the HGF expression vector (containing 10 µg/ml of the HGF expression vector). The HVJ-liposomes containing the HGF expression vector is hereinafter often referred to as HVJ-liposome-DNA.

Example 2

Administration of the HVJ-liposome containing the HGF expression vector to rats

[53] The HVJ-liposomes containing the HGF expression vector were prepared by the method ~~as~~ described in the above Example, using 64 µg of HMG 1 nuclear protein and 200 µg of plasmid DNA. BSS was added to and mixed with the liposome suspension (0.5 ml, containing 10 mg of the lipids) and HVJ (35,000 hemagglutinating units) to make the whole volume 2 ml.

[54] SD rats (weighing 400-500 g; purchased from Japan Charles River) were anesthetized with intraperitoneal administration of sodium pentobarbital (0.1 ml/100 mg), warmed and maintained breathing was ~~by~~ with an automated breather. The rats were subjected to

thoracotomy ~~at~~ on the left side. The HVJ-liposome-DNA or HVJ-liposome-cont (20 µl) was carefully injected directly through the cardiac apex using a 30 G syringe.

Comparative Example 1

Preparation of HVJ-liposomes containing no HGF expression vector

[55] A vector bearing no HGF gene was treated in the same manner as described in Example 1 to prepare the HVJ-liposomes containing no HGF expression vector. The HGF expression vector-free HVJ-liposomes are hereinafter referred to as HVJ-liposome-cont.

Comparative Example 2

Preparation of HVJ-liposomes containing human TGF-β expression vector

[56] HVJ-liposomes containing human TGF-β expression vector were prepared in a manner similar to that described in Example 1 except for using a human TGF-β expression vector.

[57] The HVJ-liposomes containing human TGF-expression vector are hereinafter referred to as HVJ-liposome-DNA (TGF-β).

Test Example 1

Expression of HGF in rat coronary endothelial cells sensitized with HVJ-liposome-DNA

[58] HVJ-liposome-DNA (concentration of the HGF expression vector in liposomes: 10 μg/ml) was sensitized to rat coronary endothelial cells (cell count: 10^6). HGF production was determined by ELISA. For control, a similar test was conducted using HVJ-liposome-cont. HGF production was also determined on the non-sensitized rat coronary endothelial cells (no-treatment group). The results are shown in Fig. 1 (n = 6), wherein "HGF" represents the group of rat coronary endothelial cells sensitized with HVJ-liposome-DNA.

[59] As shown in Fig. 1, the rat coronary endothelial cells sensitized with HVJ-liposome-DNA produced and secreted HGF ~~on~~ at a high level. On the other hand, HGF production was not substantially observed either in the intact group or in the group of ~~the~~ rat coronary endothelial cells sensitized with HVJ-liposome-cont.

[60] Cell counting in the groups tested reveal~~s~~ed that the HGF expression group showed significantly high cell counts.

Test Example 2

Effects of the sensitized HGF expression vector on proliferation of endothelial cells

[61] Human endothelial cells were sensitized with HVJ-liposome-cont. The sensitized cells were incubated in the presence or absence of added exogenously recombinant human HGF (1, 10 and 100 ng/ml) and ~~a~~ the cell

growth rate (%) was determined. The results are shown in Fig. 2 ((line) graph, n = 6), wherein "DSF" represents the group of ~~the~~ endothelial cells sensitized with HVJ-liposome-cont and "HGF" represents ~~shows~~ the group of endothelial cells incubated in the presence of recombinant human HGF in a definite concentration (*: P < 0.05, **: P < 0.01 for DSF).

[62] The (line) graph shown in Fig. 2 reveals that the growth of endothelial cells is promoted by the exogenously added HGF.

[63] The endothelial cells sensitized with HVJ-liposome-DNA (concentration: 10 μg/ml) were similarly incubated, ~~and~~ the increase~~d~~ in the number of cells ~~were counted to determine a~~ was determined, and the cell growth rate (%) was calculated. For a control, ~~the~~ endothelial cells sensitized with HVJ-liposome-cont were also incubated, ~~and~~ the increase~~d~~ in the number of cells ~~were counted to determine a~~ was determined, and the cell growth rate was calculated (%). The results are shown in Fig. 2 (bar, n = 6), wherein "DSF" designates the group of ~~the~~ endothelial cells sensitized with HVJ-liposome-cont, and "HGF" designates the group of ~~the~~ endothelial cells sensitized with HVJ-liposome-DNA (**: P < 0.01 for DSF, #: P < 0.05 for HGF, 100 ng/ml).

[64] As is noted from the bar shown in Fig. 2, the results reveal that the cell growth rate of the HVJ-liposome-DNA-sensitized endothelial cells is markedly higher than that of the control group and significantly high even when compared to that of ~~the~~ cells with exogenously added HGF.

[65] The aforesaid endothelial cells sensitized with HVJ-liposome-DNA were incubated in the presence or absence of a rabbit anti-human HGF antibody. The increase in the number of cells ~~increased were counted to~~ was determined ~~a~~ and the cell growth rate was calculated. For a control, the endothelial cells sensitized with HVJ-liposome-cont were incubated, and the increase in the number of cells ~~increased were counted~~ was determined in a similar manner in order to ~~determine~~ calculate the ~~a~~ cell growth rate. The rabbit anti-human HGF antibody (10 μg/ml) was purified by the method ~~as~~ described in Jpn. J. Cancer Res., 83, 1262-1266 (1992). This antibody is capable of neutralizing the biological activity of 10 ng/ml ~~in~~ at ~~its~~ a concentration of 10 μg/ml. The anti-human HGF antibody ~~is cross-reactive~~ reacts only with human HGF ~~but~~ and not with rat HGF, whereas the anti-rat HGF antibody ~~is cross-reactive~~ reacts only with rat HGF ~~but~~ and not with human HGF. Normal rabbit serum IgG (10 μg/ml) was used for the control.

[66] The results are shown in Fig. 3 (n = 6), wherein "control" designates the group of HVJ-liposome-cont-sensitized endothelial cells incubated in the presence of IgG control; "HGF" designates the group of HVJ-liposome-DNA-sensitized endothelial cells incubated in the presence of IgG control; and "HGFab" designates the group of HVJ-liposome-DNA-sensitized endothelial cells incubated in the presence of the rabbit anti-human HGF antibody. The cell growth rate (%) is expressed in terms of relative % when the growth rate in the control group is made 100 (*: $P < 0.01$ for the control group, #: $P < 0.05$ for HGF). As shown in Fig. 3, the growth of

HVJ-liposome-DNA-sensitized endothelial cells was arrested in the presence of ~~the~~ anti-human HGF antibody, and the cell growth rate was thus substantially the same as that of the control group. These results clearly demonstrate that HGF is the growth factor of endothelial cells.

<u>Test Example 3</u>

<u>Effects of the supernatant of the incubated HVJ-liposome-DNA-sensitized rat VSMCs on rat coronary endothelial cells</u>

[67] The supernatant from the incubated HVJ-liposome-DNA-sensitized rat VSMCs was ~~were~~ added to the rat coronary endothelial cell culture system (cell count: 10^5) during the stationary phase. After incubation ~~was conducted~~ for 3 days, ~~the count of the~~ the increase in the number of endothelial cells ~~increased~~ was determined ~~examined~~. For a control, the supernatant of the incubated HVJ-liposome-cont-sensitized rat VSMCs was added to the rat coronary endothelial cells in a ~~were treated in the~~ similar manner to that ~~as~~ described above, and the increase in the number of endothelial cells was determined ~~increased were counted~~ as described above. The results are shown in Fig. 4 (n = 6), wherein "control" indicates the group to which was added ~~with~~ the supernatant from the incubated HVJ-liposome-cont-sensitized rat VSMCs, and "HGF" represents the group to which was added ~~with~~ the supernatant of the incubated HVJ-liposome-DNA-sensitized rat VSMCs.

[68] As shown in Fig. 4, a significant increase in the number ~~count~~ of endothelial cells was observed ~~noted~~ in the group

to which was added ~~with~~ the supernatant of the incubated HVJ-liposome-DNA-sensitized rat VSMCs.

[69] The concentration of HGF in the culture supernatant of the rat VSMCs sensitized with HVJ-liposome-DNA or HVJ-liposome-cont as described above was assayed by ELISA using an anti-human HGF antibody and an anti-rat HGF antibody. The HGF concentration in the culture supernatant of non-sensitized VSMCs was also assayed (no-treatment group).

[70] The results obtained using the anti-human HGF antibody and the anti-rat HGF antibody are shown in Figs. 5 and 6, respectively (n = 6 in both tests). In the figures, "control" represents ~~designates~~ the group to which was added ~~of~~ the supernatant from the incubated HVJ-liposome-cont-sensitized rat VSMCs; and "HGF" represents ~~designates~~ the group to which was added ~~of~~ the supernatant from the incubated HVJ-liposome-DNA-sensitized rat VSMCs.

[71] As shown in Fig. 5, HGF was detected in the supernatant of the HVJ-liposome-DNA-sensitized rat VSMCs, and the HGF concentration was significantly higher than that of the control group.

[72] Fig. 6 also reveals that rat HGF was further detected in the supernatant of the HVJ-liposome-DNA-sensitized rat VSMCs, and the HGF concentration was significantly higher than that of the control group.

[73] As observed in Figs. 5 and 6, no HGF was present in an amount detectable by ELISA, in both the supernatants of the intact group and the control group.

Test Example 4

Effects of the supernatant from the incubated HVJ-liposome-DNA-sensitized rat coronary endothelial cells on rat coronary endothelial cells

[74] The supernatant of the incubated HVJ-liposome-DNA-sensitized rat coronary endothelial cells ~~were~~ was added to the rat coronary endothelial cell culture system (cell count: 10^5) during the stationary phase. After incubation for 3 days, ~~the count of~~ the increase~~d~~ in the number of endothelial cells was examined. For the control, the endothelial cells were incubated in ~~the~~ a similar manner, using ~~the~~ culture supernatant ~~of~~ from the HVJ-liposome-cont-sensitized rat coronary endothelial cells, and the increase~~d~~ in the number of endothelial cells ~~were counted~~ was determined. The results are shown in Fig. 7, wherein A, B and C represent~~s~~, respectively, the group to which was added ~~with~~ the culture supernatant of the HVJ-liposome-DNA-sensitized rat coronary endothelial cells (n = 8), the group to which was added ~~with~~ the culture supernatant of the HVJ-liposome-cont-sensitized rat coronary endothelial cells (n = 8), and the no-treatment group (n = 15).

[75] As shown in Fig. 7, a significant increase in the ~~count~~ number of endothelial cells was ~~noted~~ observed in the group to which was added ~~with~~ the culture supernatant of the HVJ-liposome-DNA-sensitized rat coronary endothelial cells, whereas in the control group, the cell count was almost the same as that of the no-treatment group (control group: 0.117 ± 0.002, group A: 0.148 ± 0.03, P < 0.01).

[76] Next, ~~an~~ anti-HGF antibody was added to the culture supernatant of the HVJ-liposome-DNA-sensitized rat coronary endothelial cells. The ~~count of~~ increase~~d~~ in the number of endothelial cells was determined ~~examined~~ as described above. The results are shown in Fig. 8 (n = 8), wherein A represents the group to which was added ~~with~~ the culture supernatant of the HVJ-liposome-DNA-sensitized rat coronary endothelial cells; B represents the group to which was added ~~with~~ the culture supernatant of the HVJ-liposome-cont-sensitized rat coronary endothelial cells; C represents the group to which was added ~~with the~~ anti-HGF antibody, ~~to~~ the culture supernatant of the HVJ-liposome-DNA-sensitized rat coronary endothelial cells; and D represents the group to which was added ~~with~~ a control antibody to the supernatant from the incubated HVJ-liposome-DNA-sensitized rat coronary endothelial cells, and

[77] As shown in Fig. 8, A and C, the cell growth promoting activity of the culture supernatant of the HVJ-liposome-DNA-sensitized rat coronary endothelial cells completely disappeared by adding the anti-HGF antibody thereto. The results reveal that the cell growth promoting activity of the culture supernatant of the HVJ-liposome-DNA-sensitized rat coronary endothelial cells is attribut~~ed~~able to HGF.

Test Example 5

Effects of HVJ-liposome-DNA-sensitized human VSMCs on human endothelial cells

Human VSMCs were inoculated on a cell culture

[78]

insert (manufactured by Coaster, pore diameter of 0.45 µm), ~~which were~~ and then grown in DMEM medium supplemented with 10% bovine serum. On the other hand, human endothelial cells were inoculated on a 6-well plate and maintained in DMEM medium supplemented with 10% bovine serum. When the VSMCs ~~were proliferated to~~ reached 80% confluence, the VSMCs were incubated at 4°C for 5 minutes and at 37°C for 30 minutes together with HVJ-liposome-DNA (DNA content in the liposomes: 10 µg) or with HVJ-liposome-cont. After sensitization, the insert containing the sensitized VSMCs was added to each well containing human endothelial cells in the stationary phase. VSMCs and the endothelial cells were co-incubated for 3 days in DMEM medium supplemented with 0.5% bovine serum. Thereafter the cell count was determined with a WST-cell counter kit (manufactured by Wako Co.). The results are shown in Fig. 9 (n = 6). In the figure, "control" represents the group co-incubated with the HVJ-liposome-cont-sensitized VSMCs, and "HGF" represents the group ~~of the supernatant from the~~ with the co-incubated HVJ-liposome-DNA-sensitized VSMCs.

[79] The results shown in Fig. 9 reveal that human VSMCs sensitized with HVJ-liposome-DNA could significantly increase the growth of non-sensitized human endothelial cells in the stationary phase.

Test Example 6

Effects of the HVJ-liposome-DNA-sensitized rat VSMCs on rat coronary endothelial cells

[80] The HVJ-liposome-DNA-sensitized rat VSMCs (cell count: 10^6) were co-incubated for 3 days with rat coronary endothelial cells (cell count: 10^5) in the stationary phase. Thereafter, the increase in ~~count of~~ the number of ~~increased~~ endothelial cells was determined ~~examined~~. For the control, endothelial cells were co-incubated in a ~~the~~ similar manner using the HVJ-liposome-cont-sensitized rat VSMCs, and the increase~~d~~ in the number of endothelial cells was determined ~~were counted~~. The results are shown in Fig. 10 (n = 6), wherein "control" represents the rat VSMCs group sensitized with the HVJ-liposome-DNA, and "HGF" represents the rat VSMCs group sensitized with HVJ-liposome-cont.

[81] As shown in Fig. 10, the growth of the endothelial cells was stimulated by HGF released from the HVJ-liposome-DNA-sensitized rat VSMCs, and ~~the~~ an increase~~d~~ in the cell count was observed (control group: 0.126 ± 0.006, HGF group: 0.156 ± 0.01, $P < 0.05$).

Test Example 7

Growth of rat VSMCs sensitized with HVJ-liposome-DNA

[82] Rat VSMCs sensitized with HVJ-liposome-DNA and rat VSMCs sensitized with HVJ-liposome-cont were incubated, respectively, to ~~make~~ compar~~ison~~e ~~of~~ the increase~~d~~ in the cell counts therebetween. Sensitization with HVJ-liposome-DNA did not affect cell growth at all. The

results reveal that HGF has no cell growth promoting effect on VSMCs.

Test Example 8

Induction of angiogenesis in rat heart muscle directly injected with HVJ-liposome-DNA

[83] Rat heart muscle directly injected with HVJ-liposome-DNA, rat heart muscle directly injected with HVJ-liposome-cont and rat no-treatment heart muscle were stained with HE and Azan, respectively, and examined on a microscope to count the number of microvessels. The results are shown in Fig. 11, wherein "HGF" designates the number of microvessels in rat heart muscle directly injected with HVJ-liposome-DNA, and "control" designates the number of microvessels in rat heart muscle directly injected with HVJ-liposome-cont.

[84] As is ~~noted~~ seen from Fig. 11, the number of minute blood vessels significantly increased in the rat heart muscle injected with HVJ-liposome-DNA, as compared to ~~those of~~ that in the rat heart muscle injected with HVJ-liposome-cont and the rat no-treatment heart muscle. These results ~~reveal~~ indicate that HGF which is capable of causing ~~having the activity of growing~~ endothelial cells to grow, exhibits ~~an~~ angiogenesis activity *in vivo*.

Test Example 9

Repair of articular cartilage by directly introducing HVJ-liposome-DNA into the joint

[85] Ten week~~s~~ -old ~~aged~~ Fischer's rats were injured at the femoral intercondylaris through the subcartilage using Kirschner's wires of 1.8 mm in diameter. One week after the operation, the HVJ-liposome-DNA (100 µl/knee) prepared in Example 1 was introduced directly into the joint. For the control, the HVJ-liposome-cont prepared in Comparative Example 1 and HVJ-liposome-DNA (TGF-β) prepared in Comparative Example 2 were administered directly into the joint in the same amount. Rats were sacrificed 1, 3 and 4 weeks after introduction of these genes, etc. and the histology of the repaired sites was ~~were histologically~~ observed.

[86] As shown in Fig. 12, the results indicate that the synthesis of proteoglycan stained with Toluidine Blue was observed 3 weeks after administration of the HVJ-liposome-DNA into the joint, displaying the development of cartilage-like cells. Furthermore, as shown in Fig. 13, 4 weeks after administration of the HVJ-liposome-DNA into the joint, there was observed a tendency to further extend the area of developing cartilage-like cells, in which the synthesis of proteoglycan was observed ~~recognized~~.

[87] As shown in Fig. 14, where the HVJ-liposome-DNA (TGF-β) prepared in Comparative Example 2 was injected into the joint, development of such cartilage-

like cells was not observed even 4 weeks after ~~the~~ administration. Furthermore, as shown in Fig. 15, where the HVJ-liposome-cont prepared in Comparative Example 1 was injected into the joint, development of such cartilage-like cells was not observed even 4 weeks after ~~the~~ administration.

Industrial applicability

[88] The medicament of the present invention provides persistent therapeutic effects, as compared to HGF itself. Moreover, the medicament of the present invention may be topically applied to the target organs so that the effects can be selectively exhibited, resulting in minimizing the side effects of HGF.

WHAT IS CLAIMED IS:

1. A medicament comprising a HGF gene.

2. A liposome containing a HGF gene.

3. A liposome according to claim 2, which is a membrane fusion liposome fused to Sendai virus.

4. A medicament comprising a liposome according to claim 2 or 3.

5. A medicament according to claim 1 or 4, which is for use in the treatment for arterial diseases.

6. A medicament according to claim 1 or 4, which is for use in the treatment for cartilage injury.

ABSTRACT OF THE DISCLOSURE

The present invention relates to a medicament comprising a HGF gene. The medicament of the present invention may be topically applied to the target organs so that the effects can be selectively exhibited, resulting in minimizing the side effects of HGF.